The Biologic Diversity of Cancer Metastases

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The cellular diversity of primary tumors is now known to be associated with their metastatic potential. Thus, anticancer strategies must be carefully designed and administered to anticipate and thwart the aggressive course of metastatic disease. Phenomena underlying these pathologic mechanisms and their implications for the development of therapeutic approaches are discussed.

Although biochemical markers suggest that most tumors arise from a single cell, other criteria reveal tumors to be quite heterogeneous. The morphologic diversity of tumors was first recognized in the nineteenth century. More recently, it has been found that the cells within a single tumor may differ in terms of antigenicity, karyotype, drug sensitivity, growth rate, metabolic products, and metastatic potential.

Our laboratory has focused on determining not only the origins of this diversity but also its impact on malignancy of the tumor and its treatment. An inherent goal is development of therapeutic strategies that would succeed despite a tumor's heterogeneity.

Most significantly, we have learned that the greater a cell's metastatic potential, the less stable its phenotype. Metastatic cells, therefore, continually give rise to new variants. We also have found that solid tumors tend to reach a state of phenotypic equilibrium. If this balance is disturbed by the loss of cellular subpopulations, a new generation of tumor-cell variants emerges. As a result, anticancer therapies must be carefully designed and administered. A treatment that leaves a surviving subpopulation may trigger the generation of tumor-cell variants that are maximally refractory to available therapy.

In this article, some of the experiments that have examined the heterogeneous nature of tumor cells will be described. The review also will touch on some of the manifestations of this diversity, particularly as they relate to metastasis. The implications of these findings for the design of clinical strategies for treatment of metastases will also be discussed.

Perhaps the earliest suggestion of the significance of tumor-cell heterogeneity was by the British clinician S. Paget. In 1889, Paget proposed, in an article in *The Lancet*, the "seed and soil" hypothesis to explain the pattern of metastatic spread. Some tumors are good seeds, Paget said, whereas others are not—a simplified way of describing heterogeneity. In addition, he proposed that some organs provide good soil for a metastatic cell, whereas others do not—a way of describing one possible host-mediated selection process favoring the growth of the good seeds, i.e., the metastatic cells.

A successful metastatic cell must be able to perform a diverse array of functions. Among other abilities, the cell must be able to detach from the primary tumor, invade host tissues and gain entrance into the circulation, survive in the circulation, arrest at an organ capillary bed, extravasate, and grow in the new site (see figure, page 58). One can draw an analogy between a metastatic cell and the track-and-field athlete in the decathlon. To succeed, both must do well by a number of different criteria. In addition, decathlon champions may differ in many respects—nationality, age, size, etc. Their only common feature is that all are athletically versatile. Similarly, metastatic cells need have little in common. The only property they all must share is the ability to progress through each stage of metastasis—from event to event.

We now appreciate that the gradual selection of a metastatic cell demands that primary neoplasms be heterogeneous. What is required is that one subpopulation of cells out of many populating a tumor

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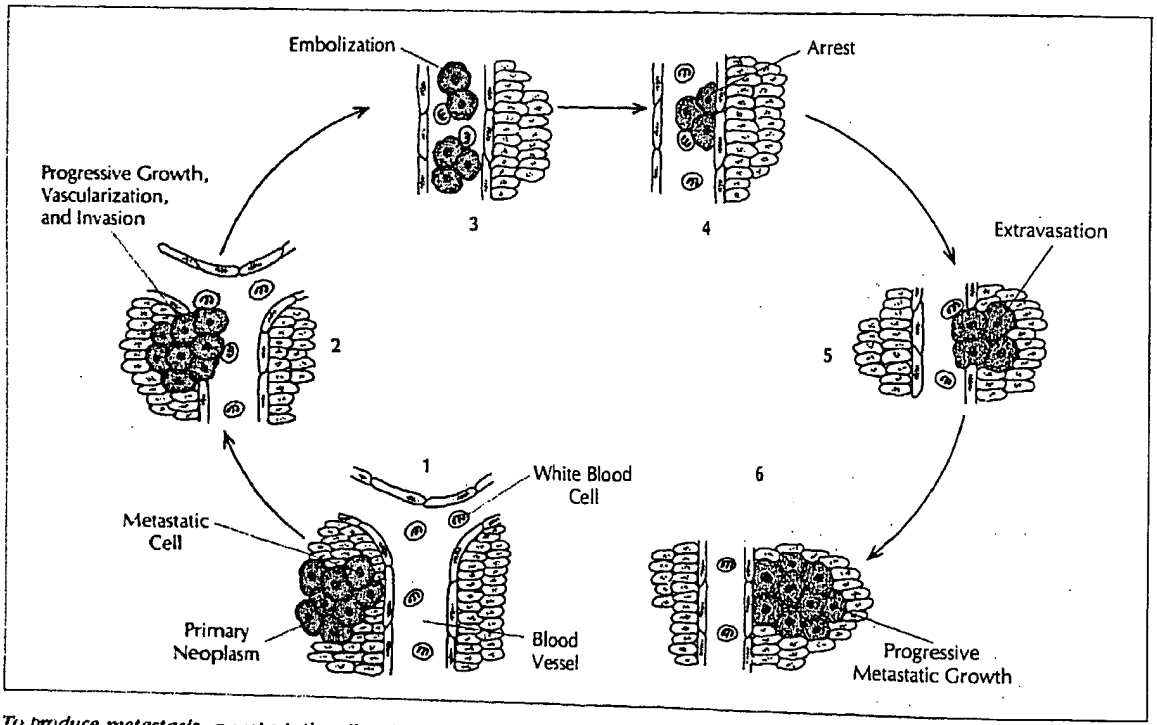
have the ability to complete one or more steps in the path toward metastasis. Such cells would replicate, of course, and among their progeny, a cell would have to appear with the ability to complete other steps of the metastatic process. This would continue until a clone (subpopulation) arises that has all the requisite properties. Obviously, these attributes could develop out of sequence and faster than one at a time. Later in this article, we shall summarize what we know about the origins of tumor-cell heterogeneity, but first, an explanation of how we came to discover that tumor cells are indeed heterogeneous for metastatic potential is in order.

In 1973, our laboratory serially selected metastatic cells until we established a line with a high metastatic potential. Our ability to perform this selection suggested that metastasis is not a random process, a hypothesis that was confirmed by an experiment performed by Fidler in collaboration with Margaret Kripke of the Cancer Biology Program in 1977. The study used as a model the classic Luria-Delbrück fluctuation test, which determines whether a mutant preexists in the population or appears as a result of "pressures" on a cell population consequent to the experimental design.

A cell culture of the B16 melanoma was established from a subcutaneous tumor growing in a syngeneic C57BL/6 mouse and was divided into two aliquots. One aliquot was maintained as a mass culture; the other was cloned to produce several cell lines, each one established from a single cell of origin. After incubation for the same period of time, equal numbers of tumor cells from each of the cloned lines and from the unselected parental line were injected in suspension into the circulation of different syngeneic mice. The groups of animals injected with the uncloned, parental line all exhibited a similar number of lung metastases, whereas the cloned sublines differed markedly from the parent tumor and among themselves in the numbers of metastases they produced (see figure, pages 60, 61). Control subcloning experiments showed that this variability was not introduced by the process of in vitro cloning. Thus, using the Luria-Delbrück assay criteria, we demonstrated that the parental tumor was heterogeneous and that populations of cells with differing metastatic capacity preexisted within the original tumor.

The B16 melanoma arose spontaneously in a mouse in 1954 and since then has been repeatedly passaged for many times the life span of its natural host. The observed metastatic heterogeneity of this tumor could be an artifact resulting from its long history. However, exactly comparable data have now



To produce metastasis, a metastatic cell proliferating within a primary tumor must survive a series of events that destroy a majority of tumor cells. The tumor must become vascularized, not only to obtain nutrients but to gain access to the host's circulation. There the metastatic cells must withstand turbulence and host defense mechanisms. Formation of a metastasis requires arrest of a metastatic embolus at an organ capillary bed, extravasation, and growth at new site.

been obtained with another murine melanoma of much more recent origin. Kripke has described the induction and isolation of a new melanoma syngeneic to the C3H/HeN mouse. The primary K-1735 melanoma was established in culture after a single transfer in an immunodeficient mouse. Cells from the fifth in vitro passage were used to produce clones. The clones and the parent melanoma were then analyzed for metastatic capacity. Here again, the clones differed dramatically from each other and from the parent tumor in their production of metastases in the lungs, lymph nodes, and other organs. Statistical analysis indicated that 20 of 22 K-1735 clones were significantly different from the parent tumor with regard to metastatic capacity.

An in vivo analysis confirmed these results. The newly developed parental cell line forms lung metastases that vary in size, shape, and pigmentation. If individual metastatic foci are selected, dispersed, and injected into fresh mice, each focus gives rise to a generation of foci that are homogeneous (see figure, page 64).

These data show that tumors of recent origin are no less heterogeneous with regard to metastatic capacity than the B16 melanoma. Longevity of neoplasms, therefore, is not a prerequisite for the generation of biologic heterogeneity.

Indeed, in an experiment performed with Ian Hart, we have determined how soon diversity can form in developing tumors. Individual embryonic BALB/c fibroblasts were transformed in vitro with a murine sarcoma virus. By 42 days after transformation, we could isolate tumor colonies, each derived from a single transformed cell, with metastatic properties that differed from the parental line.

Metastatic heterogeneity is not unique to mouse melanomas. A comparable finding has been reported for tumors of diverse histologic origin from mice, guinea pigs, rats, hamsters, and even chickens. The analysis of human tumors for metastatic diversity has been hindered by the absence of an in vivo experimental system. Recently, however, it has been found that malignant nonmurine cells will metastasize if they are injected into three-week-old athymic mice. Nabil Hanna of Smith Kline & French Laboratories has used this system to confirm that primary human tumors also display extensive metastatic heterogeneity.

When discussing tumor-cell heterogeneity, it is, of course, important to specify parameters. We have chosen to examine diversity in metastatic potential and resistance to cytotoxic agents because these properties are important in the clinical management of cancer. Tumor cells, however, may display heterogeneity for a variety of other properties. For this reason, a selection process that results in cellular homogeneity for one character need not eliminate a population's heterogeneity for other properties.

Recognition of this is important when evaluating recent work that, for example, has attempted to identify biochemical markers that can be used to gauge the progressive growth of a tumor. Other research has attempted to develop antibodies that will bind selectively to tumor cells of a particular sort. We have come to question the value of these approaches since our results clearly indicate that there are few properties that can be attributed with confidence to every tumor cell of a particular sort.

The only properties that surely can be found in malignant cells are those that contribute directly to malignancy. Such properties allow a cell to progress from a state of dependency on the host to one of growth independent of host control. If, for example, a tumor cell is dependent on exogenous estrogen for its growth, it will be autonomous only when it no longer requires estrogen. Similarly, an autonomous cell must produce growth factors and evade host defense mechanisms. The progression or evolution of tumors from the benign to the malignant state is due to cumulative effects of such requirements.

This concept that malignant cells appear as the result of progressive changes was first introduced in 1954 by Leslie Foulds of the Institute for Cancer Research in London. The concept was refined in 1976 by Peter Nowell of the University of Pennsylvania School of Medicine, who suggested that the process of tumor progression could be the result of a genetic mechanism. Tumor cells, Nowell said, are less genetically stable than normal cells, and therefore, they do not replicate accurately. This causes the emergence of new variants that are better able to grow free of the controls that regulate the growth of normal cells or even benign tumor cells. In this way, a series of independent intracellular changes result in clonal subpopulations within a tumor. One prediction of this hypothesis is that cells with a higher metastatic potential (more progressive nature) should have a more unstable phenotype than cells with a lower metastatic potential (less progressive nature). This instability would be a by-product of the heritage of multiple mutations that produced the highly metastatic cell.

This prediction has been tested in collaboration with Maria Cifone, using cells from UV-2237, a malignant murine fibrosarcoma that produces lung metastases. We first assessed the stability of the metastatic phenotype in clonal lines exhibiting high or low metastatic potential. The clonal line with low metastatic potential was grown for 72 days; the line with high metastatic potential was grown for 60 days. One part of each cell type was passaged in vitro, and a counterpart of the population was grown in vivo as a subcutaneous implant.

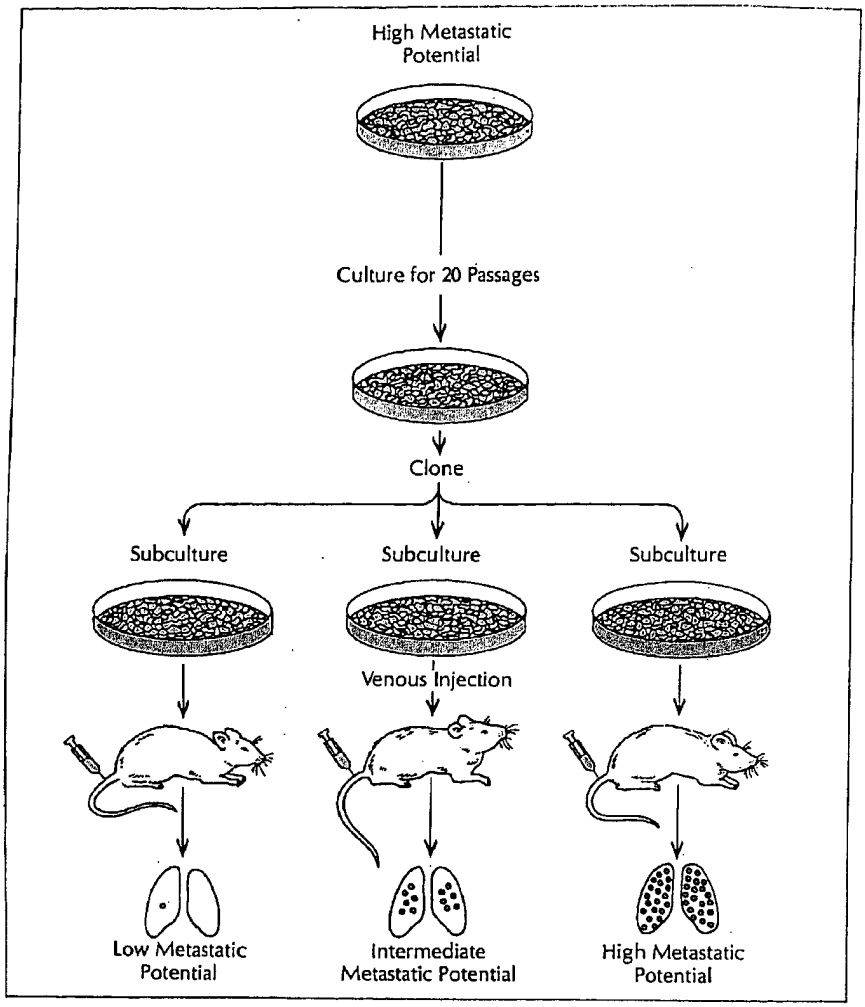
At the end of these growth periods, subclones were isolated and evaluated for their ability to produce lung metastases. In both the in vivo and in vitro systems, the clone with a low metastatic potential retained its phenotype of low metastatic potential through the 72 days of growth, during which about 85 cell doublings occurred. In contrast, after 60 days of growth, the clone with a high metastatic

neighboring cells, the stabilizing influences disappear and the cloned line diversifies to give rise to variant cells until a new equilibrium is achieved.

We tested the instability of clonal populations in the following experiments. Clones from the B16-F10 line were selected and cultured for 10, 20, or 40 in vitro passages. At each of these end points, subclones were isolated and tested for their metastatic potential. We found that after as few as 10 passages, five weeks after the clonal lines were begun, cells with divergent metastatic properties emerged. After 20 passages, 10 weeks after cloning, a majority of the subclones had a metastatic potential that was different from that of the parental clone. These differences were increased after 40 passages (20 weeks). Growth of B16-F10 clones in vivo produced a similar result, as did clones derived from B16-F1 cells, a line with a lower metastatic potential.

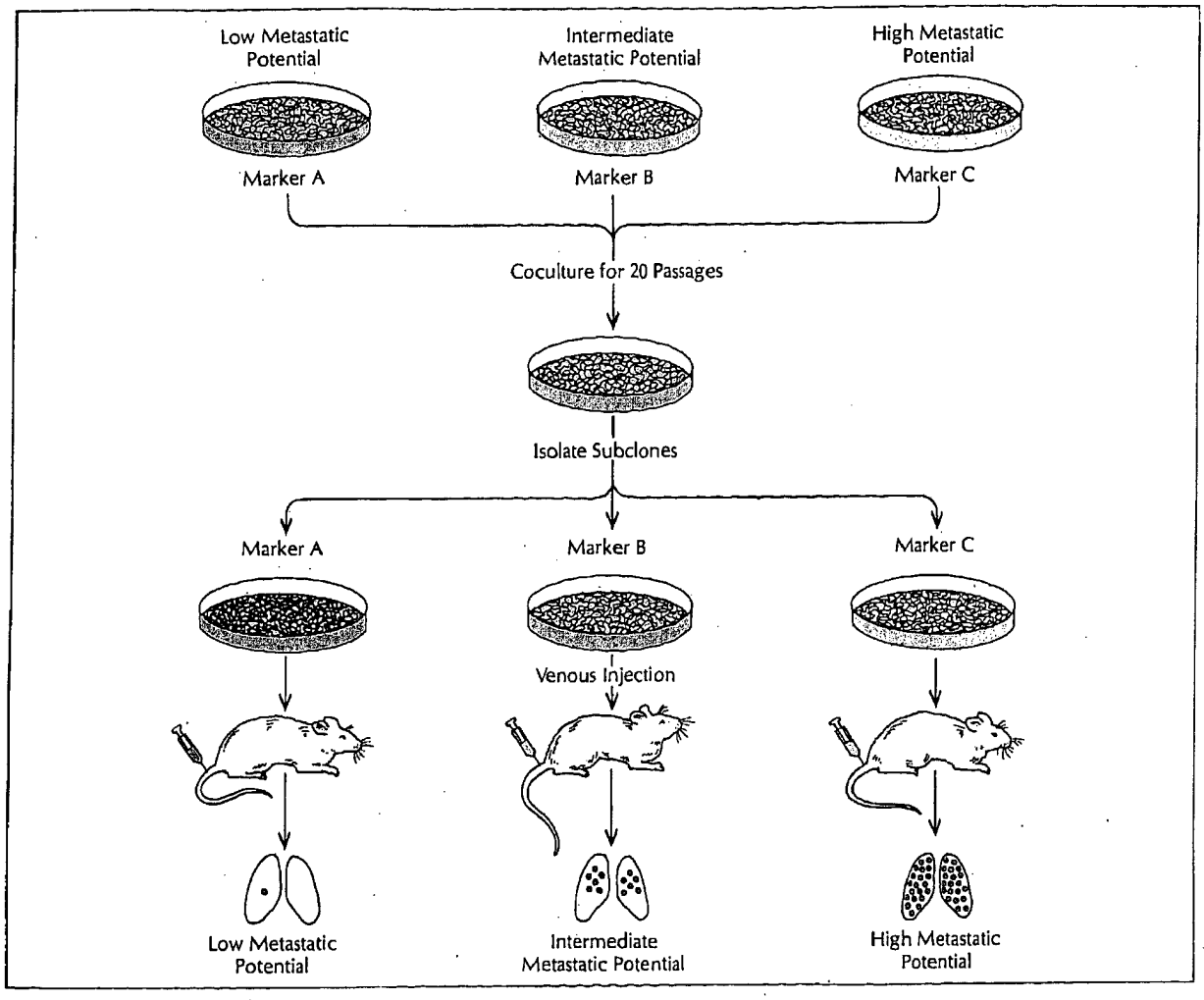
We then isolated three B16-F10 clonal lines—one with a high metastatic potential, one with an intermediate potential, and one with a low potential—and mixed them into a single culture. Each of these clonal lines had been selected to have an identifying biochemical marker. The mixed cells were passaged 20 times, and subclones were then isolated and tested for both biochemical marker and metastatic potential. Each subclone was as metastatic as its parent line, indicating that the cocultivation of several cell types, each with a different metastatic potential, somehow exerted a stabilizing influence on all the cells (see figure above). This influence appears to be specific for cells derived from the same tumor: When clones with differing lineages were cocultivated, no stabilizing effect could be observed.

We also examined what happens to the stabilizing influence of cocultivation when a selection pressure is placed on the mixed population. A diverse culture was established that was similar to the one described earlier, except that each clone used was also resistant to a specific drug. As before, the mixed populations maintained their phenotypic stability. When a drug was added that killed most of the constituent cells, the surviving, drug-resistant population began to produce metastatic variants. If a new drug-resistant clone was added, phenotypic stability returned to the culture. In the face of a new selection pressure, however—specifically, the addition of another drug—the surviving cells again became phenotypically unstable and thus diversified.



Upon cloning, a subpopulation of metastatic cells becomes phenotypically unstable. Following 20 passages, the clonal line is composed of cells with a wide range of metastatic potentials (above). If clonal subpopulations are cocultivated, their phenotypic

Our results have led to the following picture of tumor-cell evolution: When a change occurs in a cell that allows it to grow autonomously, the cell's proliferatation is enhanced as a result of its growth advantage. Those cells that become endowed with many growth-enhancing properties are eventually able to metastasize or to overgrow the tumor. As a result of this origin, metastatic cells are also, relatively speaking, phenotypically unstable. As long as the metastatic cells remain part of a heterogeneous tumor, their individual instability is held somewhat in check. When metastasis occurs, leading to



stability is maintained. To demonstrate this, three clonal populations with different metastatic potentials and different biochemical markers were grown in a single culture for 20 passages. Cells of each parental clone were then isolated according to the markers they carried. Each subpopulation had retained its original metastatic potential.

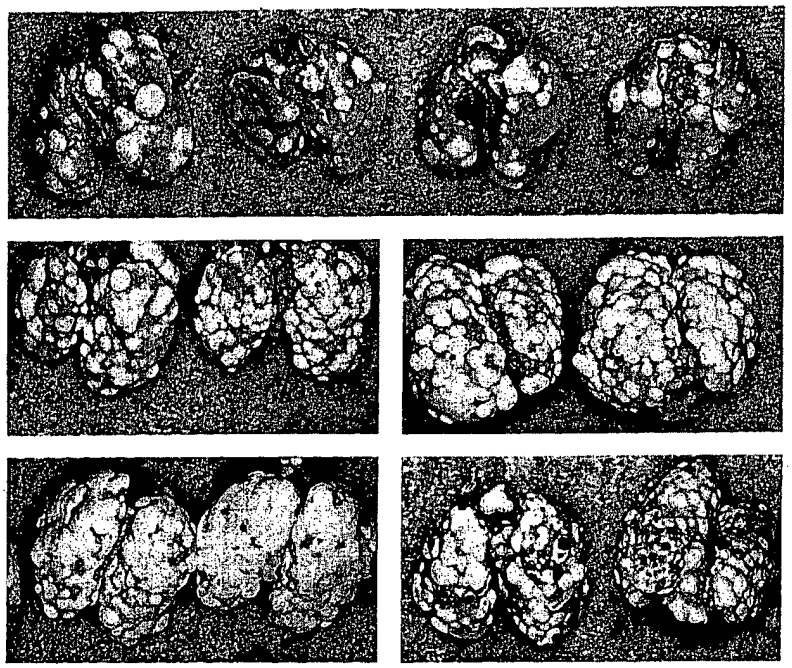
the formation of a clonal colony, the stabilizing influences are temporarily removed and variants can appear with increased frequency until a new, local equilibrium is established. This equilibrium, however, is fragile. If it is disrupted—for example, by selective cell killing by drugs—the surviving cells undergo a new phase of instability, which can give rise to a new generation of variant tumor cells.

It must be stressed that the investigations described here have dealt with a limited number of tumor systems, all of which were derived from solid tumors. We cannot say to what extent, if any, our results apply to the behavior of cells from leukemias, lymphomas, or other types of solid tumor. One priority in our research is to establish whether our findings have a limited or general significance. Although the experiments required to resolve this question are conceptually straightforward, they will require a large commitment of time and resources.

The implications of our findings for tumor therapy are potentially both significant and disheartening. Any treatment that leaves a sizable surviving population is likely to be ineffective in the eradication of cancer. A tumor that is 1 cm in diameter, perhaps the smallest that can be diagnosed clinically, contains 10^9 cells. Even if a treatment kills 99% of these cells, there will be 10^7 survivors. From this population, variants will develop that may well be more resistant to treatment than the cells in the original tumor.

In light of this, what is an oncologist to do?

We can make certain recommendations that could be applied to current therapeutic regimens. When dealing with a cancer that one knows, on the basis of clinical ex-



When cells from the parental K-1735 melanoma are injected into mice, they produce lung tumors that are heterogeneous in size, shape, pigmentation, etc (top). If cells from newly developed lung metastases are injected into mice, however, cells from each metastatic line produce foci that appear homogeneous, although differing from each other, implying that the cells in each metastatic focus are more uniform.

perience, has a high probability of metastasis, the physician should as far as possible treat "expectantly." Thus, institution of, for example, a chemotherapeutic regimen for metastatic disease should not await absolute clinical proof of recurrence. By the time a metastasis is detected, all too often it has generated so many cell variants that it is virtually impossible to treat with conventional agents.

In treating metastatic disease, the design of a therapeutic regimen should be based on the characteristics of the secondary tumors rather than on the primary tumor, if at all possible. For example, when using an in vitro cytotoxicity test to select drugs to be employed in therapy, the physician should try to concentrate on using metastatic cells. It is quite possible that drug-resistant mutants have appeared that are not even represented in most of the metastatic foci, but any of the metastatic cells are more representative of the drug's target than the cells in the primary tumor. A clinician must keep in mind that the first priority of anticancer therapy is to kill cells with metastatic potential. These cells present the greatest threat to the patient.

A further recommendation is that the individual drugs not be used sequentially. Instead, drugs should be administered in combination. This approach should maximize the effect of treatment.

Due to the toxic nature of most anticancer drugs, it may be very difficult to sustain such a barrage. We therefore have been investigating an additional anticancer therapy, which is based on the systemic activation of tumoricidal properties in macrophages. For reasons that are currently unknown, macrophages appear able to discriminate efficiently between neoplastic and normal cells and preferentially to lyse tumorigenic cells. This recognition of tumor cells also seems to transcend diversity. At least in vitro, macrophages recognize and destroy tumor cells regardless of the variations they may display. Although we can select tumor cells that are resistant to other immune system components, we so far have been unable to select tumor cells that are resistant to macrophages.

We have been devising methods by which the activity of macrophages can be enhanced in vivo. So far, we have had encouraging success in mice, but such therapy is still years away from being tried in humans. Perhaps, however, by the end of the decade, a method by which virtually every tumor cell in a patient can be safely destroyed, regardless of diversity, may be at hand. □

Suggested Reading

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